# ASSAY OF PROTEOLYTIC DEPILATORY ENZYMES ON KERATIN; ACTIVATION WITH METABISULFITE*)

BY

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(with 2 figs.)

## Introduction

This laboratory has been engaged in a continuing program to develop a process for loosening the hair and epidermis of cattlehides by means of enzymes, as a preliminary step in leather manufacture. There is need for improving the current practice of using strong lime liquors "sharpened" by the addition of sulfides or other unhairing accelerators with high oxygen demand. Alleviation of a serious waste disposal problem is probably the principal advantage of an enzyme process, although other factors may eventually encourage its acceptance by industry.

Early in this work it was found ([1])) that a number of different proteolytic enzymes could be used ,and some of the variables were investigated. A later report ([2])) described our first attempts to correlate standard assay values with hair-loosening performance, by measuring dextrinizing action against starch and proteolysis of casein. Following this, gelatin digestion was measured by formol titration ([3])), and a more comprehensive gelatinase study by three different methods was completed ([4])). Also, the effects on grain-layer elastin were investigated by a histological technique ([5])), and compared with results by gravimetric and formol titration procedures in relation to depilation ([6])).

Besides providing a dependable means for measuring enzyme potency in the unhairing studies, direct correlation of assay results

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with depilatory activity should provide basic information on the nature of the principal substrate. The work of BOSE et al.[7]) implicated the skin mucoids. Histochemical observations ([8])) tend to confirm this, and point to the dermo-epidermal junction as the site of cleavage. But the actual mechanism involved in disengaging the epidermis from underlying dermis remains obscure. Some form of proteolysis appears to be essential, yet the relationship between protease assays and depilatory action is not consistent enough to permit dependable predictions or conclusions.

This report is concerned with an assay against keratin in the form of chopped wool. Keratin is the distinctive protein of epidermal tissue. It is directly involved in the mechanism of lime-sulfide depilation, although most of the available evidence (including this report) does not indicate a similar role in the enzymic mechanism.

## Materials and Methods

The suppliers and biological sources of most of the enzymes have been listed previously ([6])). Keratinase, produced by *Streptomyces fradiae*, was obtained from the Institute of Microbiology, Rutgers University, New Brunswick, New Jersey*). Crystalline trypsin and its specific inhibitor came from Nutritional Biochemicals Corp., Cleveland, Ohio. All products were used directly without further treatment.

The wool used as substrate was a special grade of medium, commercial, scoured yarn**). Before use it was soaked for one-hour periods in 3 changes of distilled water at 36° C, air-dried and cut into small fragments about 2 to 4 mm long.

Tris (hydroxymethyl) aminomethane, at a final concentration of 0.05 M, was used as buffer from pH = 6 to 9.5, adjusting to the desired pH with HCl or NaOH. At pH = 5.5, 0.025 M $K_2HPO_4$ was used instead, adjusting with $H_3PO_4$.

The assay method was originally developed by a group at the Institute of Microbiology under the direction of Dr. W. J. NICKERSON, in connection with their discovery ([9])) of the keratinase enzyme. In his thesis, NOVAL[10]) described the responsible organism and gave results of its outstanding action on keratin, with additional information appearing in a subsequent publication ([11])). Our procedure, slightly modified from the original, used 450 mg chopped wool mixed (by stirring rod) with 20 ml of buffered enzyme in a 2 cm test tube. Appropriate blanks were included to measure separately the absorbing material contributed by enzyme and substrate alone. All tubes, in duplicate, were incubated in a water bath at 36°C for 3 hours, then filtered to stop the reaction and clarify the solutions.

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*) The enzyme is currently being produced experimentally by Merck & Co., Inc., Rahway, N.J., for possible commercial development.

**) Described as 64s, 50:50 blend of Australian: Penna./Ohio, supplied as 5 g skeins by Testfabrics, Inc., New York.

Material released from the wool was determined by measuring, in a Beckman DU Spectrophotometer, the absorbance of each filtrate at 280 mμ as suggested by NOVAL[10]). Later it was noted that the actual absorption peak was closer to 275 mμ. This wavelength was therefore used in the activation studies. Corrected absorbance values, representing the increase due to interaction of enzyme and wool, were converted to keratinase units (1 KU = 0.040 corrected absorbance) and expressed as KU per mg of enzyme.

Standard curves obtained by plotting log corrected absorbance vs. log enzyme concentration ([10])) showed a linear relationship, in most cases, from about 5 to 30 μg/ml. Typically the curve broke rather sharply above 50 μg and completely reversed near 100 μg. For this reason, and to make results comparable, all reported values are based on a constant enzyme level of 20 μg/ml.

## Experimental Results

### Survey of Enzymes:

A representative group of enzymes, selected from a large collection, was assayed in the manner described. Test results, as well as the natural pH and absorbance of each product, are shown in Table I. It can be seen that keratinase had the most significant activity under these conditions. Also, if values below 10 KU/mg are considered negative, it is apparent that optimum activity occurred at the highest pH tested (8.5) in each case, with essentially no activity at the lowest pH (5.5). It is interesting to compare results among the three tryptic products: although ranging from crude Viokase (defatted whole pancreas) to crystalline trypsin, they all showed the same low degree of activity. Among the five closely related bacterial preparations (HT) there was a somewhat wider range of activity. The plant enzymes bromelin and papain and a fungal enzyme (Rhozyme P-11), among others, were inactive.

### Metabisulfite Activation:

When bromelin and papain were found to be inactive, it was anticipated that they would probably respond to activation by one or more reducing agents. Activation of papain by $H_2S$ had been known since 1910 ([12])), and it had been suggested ([13])) that enzymatic unhairing with papain was assisted by the addition of bisulfite. MIDDLEBROOK and PHILLIPS[14]) devised a commercial process for altering wool properties with papain and bisulfite at neutral pH. BLACKBURN[15]) showed that papain with bisulfite (at 65 °C) could decompose wool into cortical cells and a soluble component. BISERTE and PIGACHE[16]) described wool digestion by pepsin-bisulfite at 37 °C and later ([17])) found that papain was more active. Consequently we did some preliminary tests with papain and bromelin. Using

TABLE I.

Survey of Selected Enzymes Showing Effect of pH on Partial Digestion of Wool.

| Enzyme product | Enzyme property | | Enzyme action on wool | | |
|---|---|---|---|---|---|
| | natural pH* | Absorbance** | pH = 5.5 | pH = 7.0 | pH = 8.5 |
| | | | KU/mg | KU/mg | KU/mg |
| Keratinase (K5A) | 5.7 | 0.024 | 15.0 | 76.9 | 168 |
| HT Proteolytic (416) | 7.7 | 0.009 | 6.3 | 47.5 | 61.3 |
| HT Proteolytic (110) | 7.6 | 0.015 | 0 | 30.0 | 38.8 |
| HT Concentrate (4903) | 7.8 | 0.022 | 3.8 | 26.3 | 47.5 |
| Protease 15 Conc. | 7.3 | 0.019 | 0 | 11.3 | 23.8 |
| Enzyme 4511-3 | 7.5 | 0.011 | 6.3 | 0 | 18.8 |
| Rhozyme P-11 | 7.1 | 0.007 | 0 | 7.5 | 0 |
| Viokase | 6.0 | 0.044 | 8.8 | 12.5 | 31.3 |
| Trypsin (noncryst.) | 6.3 | 0.023 | 0 | 8.2 | 23.8 |
| Trypsin (cryst.) | 4.2 | 0.013 | 0 | 13.8 | 22.5 |
| Bromelin | 5.3 | 0.019 | 0 | 0 | 0 |
| Papain | 5.6 | 0.012 | 0 | 0 | 0 |
| HT Conc. "P" | 7.6 | 0.018 | — | 33.8 | — |
| Protease L-56-D | 6.9 | 0.015 | — | 17.5 | — |
| Rhozyme H-39 | 7.9 | 0.007 | — | 16.9 | — |
| Prolase 40 | 6.4 | 0.003 | — | 10.0 | — |
| Special Diastase 160 | 6.9 | 0.009 | — | 7.5 | — |
| HT Conc. "A" | 7.9 | 0.028 | — | 0 | — |
| Lipase B | 7.0 | 0.013 | — | 0 | — |

*) Aqueous 0.2% stock solution.

**) Enzyme (20 μg/ml) in pH = 7 buffer; absorbance at 280 mμ in 1 cm cell vs. buffer.

1% sodium metabisulfite ($Na_2S_2O_5$) in the buffers, but otherwise performing the standard assay, it was found that papain showed about 51 KU/mg and bromelin 105 KU/mg at pH = 7.0, decreasing slightly at pH = 8.5. Results at pH = 5.5 were considerably lower.

To test the possibility that the metabisulfite had denatured (reduced) the wool enough to increase its enzyme susceptibility, portions of wool were pretreated under these same conditions of pH, time, temperature and metabisulfite concentration. After thorough washing and air-drying, the pretreated samples were subjected to enzyme (papain and bromelin) action by the standard assay. Again there was no significant activity by either enzyme at any pH, indicating more strongly that we were dealing with a true activation effect*). Further confirmation resulted from the following observations: a. substrate blanks increased very slowly with

*) The possibility of subsequent oxidative reversal to its original state, during the washing and drying of the pretreated wool, was not investigated.

increase in metabisulfite; b. absorption curves for enzyme blanks showed characteristic changes (below 270 mμ) in the presence of activator, suggesting a primary effect on the enzyme; and c. as shown in the following section, a distinct metabisulfite concentration optimum was evident at a given pH, rather than a constant slope that might be expected from progressive chemical degradation of substrate.

When these studies were extended to other enzymes, it was discovered that keratinase and Viokase were similarly activated, while four others responded very weakly or not at all. A detailed series of assays was then conducted to determine more accurately the interdependence of pH and metabisulfite concentration for optimum activity by four selected enzymes. Results are summarized graphically in Fig. 1, where the height of each bar represents the maximum activity obtained at the pH indicated. Sub-optimum values are not shown, but in each case enough data were obtained to determine the optimum amount of metabisulfite, as illustrated by the following representative results at pH = 7:

| Molar conc. of Na metabisulfite | Enzyme Activity, KU/mg | |
|---|---|---|
| | Keratinase | Trypsin |
| 0 | 128 | 28.8 |
| 0.01 | 311 | 136 |
| 0.03 | 349 | 276 |
| 0.05 | 316 | 299 |
| 0.07 | 269 | 226 |

With optimum activation the patterns displayed by keratinase and trypsin (in the figure) showed a striking similarity. There was, however, on the average a 2.5-fold increase in activity of keratinase, while that of trypsin was about 10-fold, due to their difference without activator. Optimum pH for these two enzymes was about 9, for the HT Proteolytic about 8 and for bromelin close to 7.

### Effect of Prolonged Incubation:

It seemed of interest to investigate the effect of time, in connection with the observed activation, in order to evaluate more fully the rate and extent of enzyme action on wool. Standard assays at pH = 9 were performed with keratinase at the usual level; separate sets were incubated for 8, 16 and 24 hours for comparison with the 3-hour value previously obtained. Parallel sets of samples activated with 0.01 M metabisulfite were run at the same time. Assay results are plotted in Fig. 2, with corresponding values for trypsin at 3 and

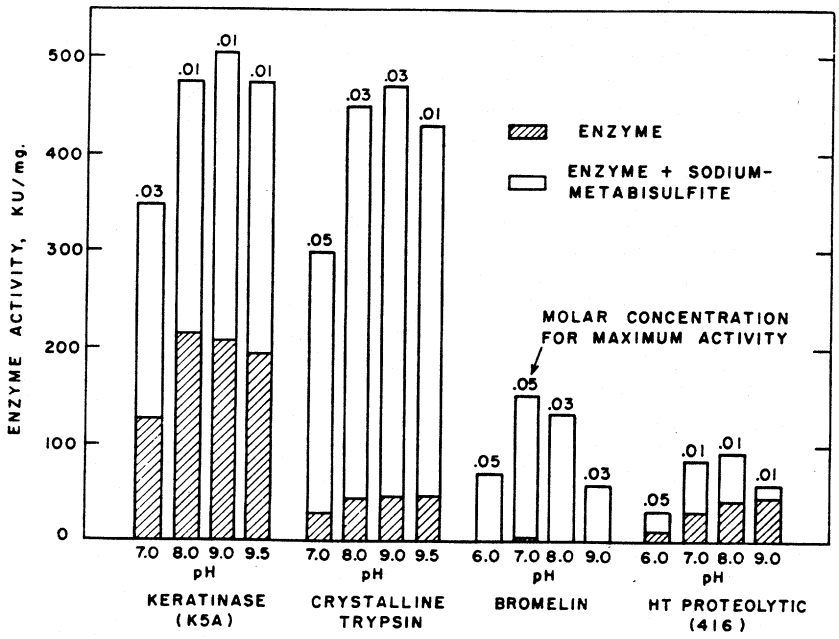


**Fig. 1.** Activation effect of metabisulfite on digestion of wool by four enzymes, as related to pH and concentration of activator (standard assay conditions).

24 hours only. With keratinase alone, the initial rate fell off slightly after 4 to 5 hours, but maintained its secondary slope until about 18 hours before dropping more rapidly. NOVAL[10]) reported the rate to be linear for only 5 to 6 hours. The activated keratinase did show an abrupt change in rate at about 3 hours, but again held its secondary rate until about 18 hours. The curves for trypsin, although incomplete, indicate a significant difference between the two enzymes.

## Effect of Other Agents:

In a brief search for additional activators, several thiol compounds and reducing agents (and a specific inhibitor) were tested for their effects on keratinase and trypsin at pH = 8.0 in the usual manner. Some of the results obtained are shown in Table II. The control value, for enzyme alone, was used to calculate the percent change recorded for each test. Cysteine appeared to activate both enzymes very strongly, but the abnormally high values for the substrate blanks make this conclusion suspect. At concentrations of 0.01 M

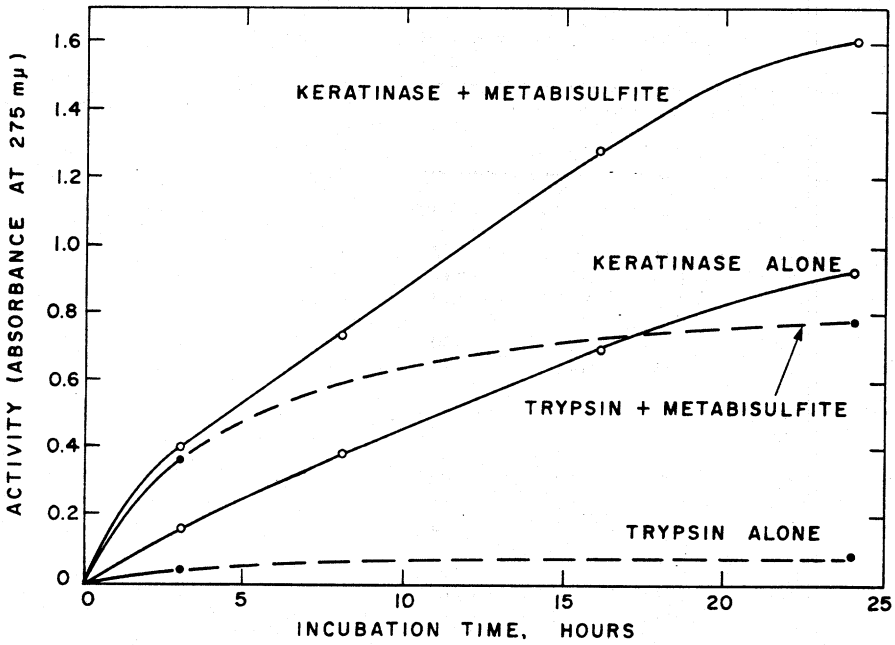


**Fig. 2.** Enzyme activity at pH = 9, with and without activator, as a function of incubation time.

and 0.05 M, cysteine blanks gave absorbance of 0.147 and 0.383 respectively; corresponding metabisulfite blanks were 0.078 and 0.098, while the control without activator was 0.035. Despite the fact that results were corrected for these blanks, proper interpretation requires extreme caution. The actual extent of activation by cysteine is therefore indefinite. Thioglycollate was moderately stimulating for both enzymes, but could not be tested above 0.03 M because of interference by its own strong absorption. Among other reducing agents, sodium dithionite (or hydrosulfite, $Na_2S_2O_4$) reacted much like thioglycollate, with even more interference than the former. Thiosulfate ($Na_2S_2O_3$) was weakly inhibitory toward keratinase while showing mild stimulation of trypsin. The soybean trypsin inhibitor was included to test further the apparent similarity between keratinase and trypsin, since their response to metabisulfite had shown such similar patterns (Fig. 1), while the time curves (Fig. 2) indicated otherwise. As expected, the inhibitor was strongly effective against trypsin, but only weakly so against keratinase.

TABLE II.

Activation or Inhibition of Keratinase and Trypsin by Various Agents at pH = 8.0.

| Enzyme additive | Concentration | Enzyme activity on wool | | | |
|---|---|---|---|---|---|
| | | Keratinase | | Cryst. Trypsin | |
| | | KU/mg | % change | KU/mg | % change |
| None (control) | — | 214 | — | 43 | — |
| l-Cysteine-HCl* | 0.01 M | 414 | + 93 | 160 | + 272 |
| | 0.05 M | 616 | +188 | 493 | +1047 |
| | 0.10 M | 529 | +147 | 694 | +1513 |
| Na-thioglycollate** | 0.01 M | 299 | + 40 | 99 | + 130 |
| | 0.03 M | 329 | + 54 | 148 | + 244 |
| Na-dithionite** | 0.001 M | 283 | + 32 | 68 | + 58 |
| | 0.005 M | 329 | + 54 | 100 | + 133 |
| Na-thiosulfate | 0.01 M | 206 | — 4 | 56 | + 30 |
| | 0.03 M | 159 | — 26 | 81 | + 88 |
| | 0.05 M | 114 | — 47 | 74 | + 72 |
| Soybean Trypsin Inhibitor, 5× cryst. | 0.002 % | 168 | — 21 | 6 | — 86 |
| | 0.010 % | 159 | — 26 | 0 | — 100 |
| | 0.020 % | 146 | — 32 | 0 | — 100 |
| Na-metabisulfite | 0.01 M | 475 | +122 | 225 | + 423 |
| | 0.03 M | 400 | + 87 | 450 | + 947 |

*) Since the substrate blanks were abnormally high, the nature of the apparent activation is in doubt.

**) Higher levels could not be tested because of excessive U.V. absorption by the agent itself.

## Discussion

Although results for different enzymes did not correlate with hair-loosening ability (since most of the preparations have moderate to strong depilatory activity), the wool assay has proven very useful for another purpose. It was designed for use with the keratinase enzyme, and with this product it has been used in a number of applications such as standardization during production, preparation of unhairing liquors, determination of stability under various conditions of use or storage and to predict unhairing potency. It might also serve as a more sensitive test of wool degradation, in the manner that the trypsin test has been used ([18]).

NOVAL and NICKERSON[11]) reported strong inhibition of keratinase by the chelating agents EDTA and sodium citrate, but no effect with potassium cyanide. This led them to conclude that an alkaline earth metal was essential for wool digestion; and they showed the

stimulative effect of added calcium and magnesium salts on digestion by the living culture. However, after repeated comparative tests we were unable to confirm any beneficial effect of $MgCl_2$ added to the enzyme system, as they had recommended.

The presence of a dispersing agent was known to facilitate rapid digestion of wool. LENNOX[19]) reported complete digestion in a few hours by a papain-bisulfite-urea system, and also found ([20])) that other disulfide bond reagents, such as thioglycollate and cysteine, could even exceed the action of bisulfite. The potential importance of these and many other reagents with respect to scission of the S-S bond has been thoroughly treated in a recent review ([21])). Likewise the possible occurrence of sulfhydryl-disulfide interchange reactions in proteins such as wool has been suggested ([21]) [22])). CREWTHER[18]) has pointed out that certain pretreatments of wool may cause a disordering effect that results in greater susceptibility to trypsin. From our limited data, only cysteine gave definite evidence of direct participation in liberation of the component being measured. Hence we have tentatively concluded that the pronounced effect of metabisulfite, in view of the mildness of our conditions, can be explained in terms of enzyme activation.

Some workers still maintain that keratin may play an important role in enzyme unhairing. SIMONCINI[23]) felt that the bacteria found in lime liquors were part of a combined chemical-biological mechanism. He isolated a number of cultures from commercial lime liquors and assayed their activity on three forms of modified keratin. Two of the most active organisms (strains of the genus *Bacillus*) proved to be potent unhairing agents, and it was significant that the addition of keratin to the growth medium caused an increase in unhairing potency. In a later report ([24])) on the activity of fungal enzymes, it was again found that the keratin assay was a better measure of depilatory activity than gelatin liquefaction or casein hydrolysis. Another *Bacillus sp.*, isolated from a living sheep, was recently described by MOLYNEUX[25]), who also demonstrated that the culture could actually digest native wool.

At any rate the keratinase preparation appears to be quite a unique enzyme system, as shown also by its unusually strong elastase activity ([6])), and it is possible that it can digest wool by a strictly enzymatic mechanism of reductase followed by proteinase. A similar mechanism has been proposed ([26])) for the clothes moth larva. It has also been shown that enzymatic reduction of disulfide linkages of a protein is possible ([27]) [28])). NOVAL and NICKERSON[11]) were unable to reduce cystine with culture broths from *Streptomyces fradiae*, and postulated that the reduction might take place only at the cell surface, which would help to account for the much greater activity of living cultures. However, the exact mechanism of the reaction is beyond the scope of our present interests.

## Summary

1. A procedure is described for the spectrophotometric measurement of a soluble component released by enzyme action, using chopped wool as the substrate. Screening of a group of depilatory enzymes at three pH levels revealed that only keratinase, from *Streptomyces fradiae*, had significant activity. There was no correlation between assay results for different enzymes and corresponding abilities to loosen the epidermis and hair of cattlehides.

2. An interesting activation effect by sodium metabisulfite was discovered, which applied to keratinase as well as to the tryptic and plant enzymes tested. Optimum conditions of activator concentration and pH were determined in some detail with four enzymes. A time study showed that keratinase digestion proceeds at a relatively constant rate well beyond the assay period. Comparative effects of other reagents were briefly explored, without finding any stronger activator (with the possible exception of cysteine).

## Résumé

1. Une méthode pour les mesures spectrophotométriques d'un composant soluble libéré par l'action enzymatique est décrite. Le substrat utilisé est le laine haché. Une étude sur un nombre d'enzymes dépilatoires à trois valeurs de pH a montré que seulement la kératinase de *Streptomyces fradiae* a une activité significative. Il n'y avait aucune corrélation entre les résultats des essais pour les différentes enzymes et leur pouvoir de détacher l'épiderme et les poils des peaux de boeufs.

2. Un effet intéressant d'activation du métabisulfite de sodium a été découvert. Ceci s'applique aussi bien à la kératinase qu'aux enzymes tryptiques et végétales étudiées. Les conditions optimales de concentration de l'activateur et du pH ont été déterminées en détail pour les quatre enzymes. Une étude en fonction de temps a démontré que la digestion avec la kératinase prend place à une vitesse constante pour des périodes bien au delà du temps d'essai. L'effet comparatif d'autres réactifs a été exploré sans trouver un activateur plus fort (avec l'exception possible de la cystéine).

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